Remarks

Following entry of this amendment, claims 1, 3-10 and 12-13 and 22-26 are pending. The specification is amended herein to clarify the priority claim, as requested in the Office action. Claims 12 and 24 are amended herein to correct typographical errors. The amendment to claim 12 was requested in the Office action. Claim 11 is canceled herein, without prejudice to renewal.

No new matter is added herein. Reconsideration of the application is respectfully requested in view of the foregoing amendments and following remarks. Applicants believe that the application will be in condition for allowance following entry of this amendment.

Objection to the Specification

The Office action requests that the first paragraph of the specification be amended to indicate that U.S. Patent Application No. 10/366,044 is a continuation-in-part of International Application No. PCT/US01/25507. The specification is amended herein to correct the priority claim as requested in the Office action, thus rendering the objection moot.

Claim Objections

Claim 12 is objected to for including a typographical error in line 3. Claim 12 is amended herein to correct the spelling of "recombination" in line 3.

Claim Rejections – 35 USC § 103

Claims 24-26 are rejected under 35 U.S.C. 103(a) as being unpatentable over Casanova et al., (Genesis, Vol. 32, No. 2, pages 158-160, Published Online 2/13/2002, cited in prior action; see the entire reference) in view of Lee et al., (Genomics, Vol. 73, pages 56-65, 2001, cited in a prior action; see the entire reference).

The Office action alleges that the evidence submitted in the previous Declaration under 37 C.F.R. § 1.131 was ineffective to overcome the Casanova et al. reference. Specifically, the Office action alleges that the Declaration under 37 C.F.R. § 1.131 does not provide evidence that "a nucleic acid encoding a selectable marker flanked by a second pair of recombining sites and a first recombining site was used for homologous recombination. The vectors of the declaration appear to contain only a pair of LoxP sites flanking a selectable marker. These LoxP sites

correspond to the selectable marker flanked by a pair of first recombining sites and the selectable marker flanked by a second pair of recombining sites; however they lack the first recombining sites in combination with the second recombining sites, which is required by claim 26."

Submitted herewith is a Declaration of Dr. Liu under 37 C.F.R. § 1.132. This Declaration is submitted to explain the data in the Declaration under 37 C.F.R. § 1.131. Specifically, Dr. Liu states that the results presented on pages 9 of the attached laboratory notes (submitted with the Declaration Under 37 C.F.R. §1.131, and described in the Declaration Under 37 C.F.R. §1.131 on page 4) describe the introduction of the second pair of recombining sites flanking a second selectable marker into a vector that already includes a first pair of recombining sites (in a nucleic acid sequence that can encode a protein once recombination occurs between these first recombining sites). Dr. Liu also states that the results presented on page 10 of the laboratory notes show the excision of the nucleic acid encoding the selectable marker with a second recombinase, which is specific for this second set of recombining sites. After this excision, the first pair of recombining sites (that were introduced previously) remain in the gene. Thus, there is a pair of recombining sites ("second" pair of recombining sites in claims 24-26) present in combination with a another recombining site ("first" recombining site in claims 24-26). The recombination of the two first recombining sites will produce a nucleic acid that encodes a functional protein in a plasmid Dr. Liu calls PL443. Note that the first pair of recombining sites and the second pair of recombining sites are both LoxP sites. Thus, Dr. Lee provides supporting information that the Declaration under 37 C.F.R. § 1.131 documents that method for generating vectors that utilizes a first and a second pair of recombining sites had been performed prior to February 13, 2002. Applicants note that the use of PL433 in *E. coli* was also described in the Declaration under 37 C.F.R. § 1.131.

The Declaration of Dr. Liu under 37 C.F.R. § 1.132 provides additional data which was also discussed with the Examiner on May 18, 2007. This data provides evidence that the PL443 vector, which was produced using claimed methods (which are identical to the methods described in the Declaration under 37 C.F.R. § 1.131), functioned for generating a knock-out of a gene both in *E. coli* and in mouse ES cells. Thus, the Declaration of Dr. Liu under 37 C.F.R. § 1.132 provides supporting evidence that the claimed methods were conceived prior to February 13, 2002. Thus, Casanova et al. is not available as a reference.

Lee et al. teach a de-repressible promoter (pL) operably linked to a nucleic acid encoding Beta, Exo and Gam under the control of a temperature sensitive repressor. Lee et al. teach that this method can be used to introduce the arabinose promoter operably linked to Cre, and describe the introduction of a FRT-Kan-FRT cassette into a gene in a bacterial artificial chromosome. However, Lee et al. do not suggest, or render obvious inserting a second nucleic acid encoding a selectable marker flanked by a pair of second recombining sites into a second site in the gene. In addition, Lee et al. do not suggest, nor render obvious, excising the nucleic acid encoding the selectable marker with a second recombinase specific for the second recombining sites, wherein two first recombining sites remain in the gene following excision of the nucleic acid encoding the selectable marker, and wherein recombination of the two first recombining sites produces a nucleic acid sequence that cannot be transcribed to produce a functional protein.

Reconsideration and withdrawal of the rejection are respectfully requested.

The Applicants respectfully request an interview if the Examiner has any questions about the data presented in either Declaration of Dr. Liu under 37 C.F.R. § 1.132 or the Declaration under 37 C.F.R. § 1.131. A formal written request for an interview is included herein.

Claims 24-26 are rejected under 35 U.S.C. 103(a) as allegedly being obvious over Casanova et al., (Genesis, Vol. 32, No. 2, pages 158-160, Published Online 2/13/2002) in view of Stewart et al., (U.S. Patent No. 6,355,412).

As discussed above, the Declaration Under 37 C.F.R. § 1.131 is sufficient to swear behind Casanova et al. Thus, Casanova et al. is not available as prior art.

Stewart et al. teach a method of performing homologous recombination that utilizes an inducible promoter operably linked to Rec E/T. Stewart et al. teach that homologous recombination can be performed using BACs. However, Stewart et al. do not teach, or render obvious methods for generating vectors for conditional knock-outs, let alone methods that include (1) using homologous recombination to insert a nucleic acid encoding a selectable marker flanked by a pair of first recombining sites into a first site in a gene in a bacterial artificial chromosome, wherein a vector comprises the bacterial artificial chromosome, or (2) excising the nucleic acid encoding the selectable maker with a first recombinase specific for the first recombining sites, wherein a single first recombining site remains in the gene, or using

homologous recombination to insert a nucleic acid encoding a selectable marker flanked by a pair of second recombining sites into a second site in the gene, or (3) excising the nucleic acid encoding the selectable marker with a second recombinase specific for the second recombining sites such that two first recombining sites remain in the gene following excision of the nucleic acid encoding the selectable marker, wherein recombination of the two first recombining sites produces a nucleic acid sequence that cannot be transcribed to produce a functional protein.

Reconsideration and withdrawal of the rejection are respectfully requested.

Claims 1, 3-10, 12, 13, 22 and 23 are rejected under 35 U.S.C. 103(a) as allegedly being obvious over Rajewsky et al., (J. Clin. Invest. Vol. 98, No. 3, pages 600-603, August 1996; see entire reference) in view of Lee et al., (Genomics, Vol. 73, pages 56-65, 2001, cited in a prior action; see the entire reference). Applicants respectfully disagree with this rejection.

Rajewsky et al. discloses that the genome in ES cells can be modified by conditional gene targeting using the Cre-loxP system. Rajewsky et al. describe a gene targeting vector including three loxP sites can be generated. This targeting vector includes three loxP sites, two of which flank a nucleic acid encoding a selectable marker. The targeting vector is introduced into embryonic stem cells, and endogenous homologous recombination is used so that the genome of the ES cells includes the selectable marker flanked by two loxP sites and the additional loxP site in the genome of the host ES cell. The expression of Cre in the ES cells results in recombination at the loxP sites. In some of the ES cells, the selectable marker is deleted, and two loxP sites remain. These ES cells are then used to generate mice. The mice including the two loxP sites are mated with another mouse that expresses Cre in specific cells of interest. Appropriate positioning of the LoxP sites can result in mutagenesis in those cells that express Cre, such as a knockout. It is the mating of mice produced from the protocol to different Cre-expressing animals (such as those that express Cre in only one tissue or cell type of interest) that can be used to produce mice of interest. Rajewsky et al. state "the strategy of conditional targeting of the endogenous genes that we have developed consists of flanking a target gene segment with loxP sites in ES cells by *classical gene targeting* and deleting the selection marker by transient transfection with a Cre-encoding plasmid." [emphases added, citation omitted, see page 601, second column]

Rajewsky et al. do not disclose any specific methods for generating conditional knockout vectors that utilize homologous recombination. Indeed, Rajewsky et al. suggest that classical gene targeting is effective at producing the vectors. The only homologous recombination used in the methods disclosed in Rajewsky et al. is the endogenous mechanisms of ES cells, wherein the final vector is incorporated into the genome. Thus, there is no rationale to combine Rajewsky et al. with any reference that teaches recombineering in bacterial cells.

There is no information in Rajewsky et al. with regard to methods for producing the vectors that are shown in Figure 1. There is most certainly no mention or suggestion in Rajewsky et al. to a bacterial artificial chromosome (BAC) to produce the vector, let alone to introduce a first selectable marker flanked by a pair of first recombining sites into a BAC. In addition, there is no mention or suggestion to use two pairs of recombining sites (as even the final vector only includes three identical recombining sites). There further is no mention in Rajewsky et al. that a first selectable marker is excised during the generation of the targeting vector. Indeed, in the methods disclosed in Rajewsky et al. the only selectable marker is excised using Cre after the introduction of the final targeting vector into ES cells.

The Office action further concedes (see page 12 of the Office action) that Rajewsky et al. do not teach two steps to introduce loxP sites, nor does it teach the use of homologous recombination to insert any nucleic acids into bacterial artificial chromosomes, let alone a first selectable marker flanked by a first pair of recombining sites and a second selectable marker flanked by a second pair of recombining sites. The Office action further concedes that Rajewsky et al. do not teach the use of a de-repressible promoter operably linked to a nucleic acid encoding Beta, Exo and Gam. Thus, it is unclear what would lead one of skill in the art to combine Rajewsky with any reference disclosing recombineering in bacterial cells.

Rajewsky et al. only teach (1) the production of vectors including two selectable markers and three loxP sites, wherein only two of the loxP sites flank a selectable marker; (2) the use of homologous recombination not to produce vectors but to introduce the vectors into ES cells, and (3) the production of mice from these ES cells and the mating of these mice to mice expressing Cre to produce gene knock-outs in animals. This is a far cry from the claimed methods for producing vectors.

Lee et al. teach a de-repressible promoter (pL) operably linked to a nucleic acid encoding Beta, Exo and Gam under the control of a temperature sensitive repressor. Lee et al. teach that this method can be used to introduce an inducible promoter operably linked to Cre, and describe the introduction of a FRT-Kan-FRT cassette into a gene (eno) in a bacterial artificial chromosome. The recombinase Flp is used to remove the selectable maker (Kan) leaving an inducible promoter linked to Cre and a single loxP site. The loxP site is then removed using gene targeting. The BAC, including an eno gene with the inducible promoter operably linked to Cre, was then used to produce transgenic mice.

There is no motivation or suggestion, in either Rajewsky et al. or Lee et al., to use methods for generating vectors for a conditional knock out that include the use of two selectable markers, each flanked by a pair of recombining sites.

Even if Rajewsky were improperly combined with Lee et al., one of skill in the art would only be motivated to use homologous recombination induced by the PL promoter operably linked to Beta and Exo (as described by Lee et al.), to produce a targeting construct including three recombining sites (as described by Rajewsky et al.), wherein two of these sites flank a selectable maker. However, there are a myriad of ways, each with a myriad of steps, that could be used to accomplish this goal. There is no motivation, suggestion, or inference in either Rajewsky et al. or Lee et al. to suggest or render obvious the specific steps of: (1) using homologous recombination to insert a second nucleic acid encoding a second selectable marker flanked by a pair of second recombining sites into a second site in the gene; followed by (2) excising the nucleic acid encoding the selectable marker with a second recombinase specific for the second recombining sites. In addition, there no teaching or suggestion to (3) excise the selectable marker such that the targeting vector cannot be transcribed to produce a functional protein.

On page 13, the Office action alleges that one of skill in the art would be motivated to modify the methods of generating a vector for a conditional knockout taught by Rajewsky et al. to include the lambda recombination system and BAC modification taught by Lee et al. Applicants respectfully disagree. As discussed above, Rajewsky et al. do not teach any methods for the production of targeting vectors. The Office action further alleges that it would be obvious to "modify the single step of homologous recombination of Rajewsky et al. to be two separate

steps, wherein the first loxP site is inserted by a separate homologous recombination event flowed by the site-specific recombination to remove the marker, and where a second the second pair of loxP sites flanking the marker are introduced by a homologous recombination event because the application of the method of Lee et al. would result in the same structure."

This assertion is difficult to understand. Rajewsky et al. only show the final targeting vector (step 1 of Figure 1). The only recombination event occurs in ES cells (step 2 of Fig. 1). Recombination results in the insertion of the targeting vector into the genome of ES cells (step 3 of Figure 1). Expression of Cre in the cell then results in recombination such that a selectable marker is removed and only a single recombining site is left in the genome of the ES cell. The method of Rajewsky et al. does not allow for two separate recombination events, because the recombination is solely in eukaryotic (ES) cells in a single event, using only the endogenous biological mechanisms present in the ES cells, that results in the integration of the vector into the target in an ES cells. There is no rationale for combining the teachings of Rajewsky et al. with those of Lee et al. Indeed, Lee et al. teach a phage lambda system which can be used to induce homologous recombination only in bacterial cells, and would not be of use in the ES cells described in Rajewsky et al. One of skill in the art simply has no rationale to combine the methods of Rajewsky et al. with Lee et al.

MPEP § 2143 states that the following must be present to support a finding of obviousness there must be: (1) a finding that the prior art included each element claimed, although not necessarily in a single prior art reference, with the only difference between the claimed invention and the prior art being the lack of actual combination of the elements in a single prior art reference; (2) a finding that one of ordinary skill in the art could have combined the elements as claimed by known methods, and that in combination, each element merely performs the same function as it does separately; and (3) a finding that one of ordinary skill in the art would have recognized that the results of the combination were predictable. This burden has not been met in the present case: (1) the prior art does not include each element claimed, as the prior art does not describe the introduction of a second selectable marker flanked by a pair of second recombining sites; (2) the prior art could not have combined the elements as claimed by known methods, since Rajewsky et al. only describe homologous recombination in ES cells with a targeting vector that only includes three recombining sites (two of these flanking a single selectable marker), and Lee et al. teach recombination methods that are functional only in

bacterial cells (not ES cells); and (3) there is no evidence that the results of the combination would be predictable or yield the claimed methods absent the presently pending claims (which simply cannot be used as part of the obviousness rejection).

Reconsideration and withdrawal of the rejection are respectfully requested.

Claim 1, 3-13, 22 and 23 are rejected under 35 U.S.C. 103(a) as allegedly being obvious over Rajewsky et al., (J. Clin. Invest. Vol. 98, No. 3, pages 600-603, August 1996) in view of Muyrers et al., (TRENDS in Biochemical Sciences, Vol., 26, No. 5, May 2001) and in view of Stewart et al., (U.S. Patent No. 6,355,412).

Rajewsky et al. is discussed above. As noted above, there is no rationale in Rajewsky et al. to support a combination with any reference teaching the construction of vectors in bacterial cells using recombineering.

Muyrers et al. teach that RecA and ET recombination can be used for direct subcloning and cloning, and describe the use of recombineering to introduce a DNA of interest into a vector. The Office action on page 15 states that Muyrers et al. teach recombinogenic engineering can involve two rounds of processing. Muyrers et al. do describe recombinogenic processing in two rounds. However, these steps are (1) the insertion of a *single* unit of DNA including a selectable marker (Fig. 1a); and (2) the removal of that selectable marker (Fig. 1b-1d, see also page 329, first column).

Muyrers et al. disclose that homologous recombination can be used for integration of a selectable gene, along with additional functional elements (see Fig. 1a), in order to introduce a DNA of interest into an episome in bacterial cells. Three methods for removing the selectable marker, such that the DNA of interest is left in the episome, are shown in Fig. 1b-1d. Fig. 1b provides a scenario wherein the nucleic acid includes both a selectable marker and a counterselectable marker. A second round of recombination is used to produce a DNA region free of any operational sequences. No recombining sites are utilized. Fig. 1d provides a scenario wherein restriction sites are included to flank the selectable gene. Restriction digestion and ligation is ultimately used to delete the selectable marker. No recombining sites are utilized. Fig. 1c provides a scenario wherein two recombining sites (such as for Cre or FLP) flank the selectable marker. The product that includes the selectable marker is exposed to the recombinase to delete the selectable marker. Muyrers et al. state that the use of recombining sites is highly

efficient, as noted in the Office action on page 15. However, Muyrers et al. state that although this is a highly efficient way to eliminate the selectable cassette, it leaves a "scar" of a single recombining site interior to the DNA of interest, which "can be a problem if left in protein coding or regulatory regions." Thus, Muyrers et al. *teach away* from the inclusion of recombining sites. Most certainly, Muyrers et al. do not describe any process wherein both a first selectable marker and a second selectable marker are inserted in a multi-step process, wherein finally recombination at two sets of recombining sites leads to a nucleic acid that cannot be transcribed into a functional product.

Muyrers et al. also disclose that a second round of selection/counterselection can be used "in cases in which the added DNA-of-interest is beyond the limits of oligonucleotide synthesis, it can be introduced in either a second round of selection/counterselection (Fig. 1b) or by use of a plasmid template constructed to carry the DNA-of interest beside of a selectable gene....(Fig. 1a)." This "second round" is very different from the two step process claimed in the present application. Moreover, even for this very different two step process, Muyrers et al. teach a completely different process for conducting the second step, a process that does not include the use of recombining sites.

As Muyrers et al. *teach away* from the use of recombining sites, and teach entirely different recombineering methods for introducing nucleic acids of interest, one of skill in the art would not combine Rajewsky et al. with this reference. Even if one were to make this *impermissible* combination, one of skill in the art would arrive at an entirely different process, that includes the use of a single nucleic acid encoding selection and counterselectable gene along side a selectable gene, followed by deletion of using counterselection and substitution of a DNA of interest marker into a target (Fig. 1b) to somehow produce a vector with only three recombining sites, as described in Rajewsky et al.

Stewart et al. teach a method of performing homologous recombination that utilizes an inducible promoter operably linked to Rec E/T in bacterial cells. Stewart et al. teach that homologous recombination can be performed using BACs. However, Stewart et al. do not teach methods for generating vectors for conditional knock-outs, let alone methods that include (1) using homologous recombination to insert a nucleic acid encoding a selectable marker flanked by a pair of first recombining sites into a first site in a gene in a bacterial artificial chromosome, wherein a vector comprises the bacterial artificial chromosome, or (2) excising the nucleic acid

encoding the selectable marker with a first recombinase specific for the first recombining sites, wherein a single first recombining site remains in the gene, or using homologous recombination to insert a nucleic acid encoding a selectable marker flanked by a pair of second recombining sites into a second site in the gene, or (3) excising the nucleic acid encoding the selectable marker with a second recombinase specific for the second recombining sites such that two first recombining sites remain in the gene following excision of the nucleic acid encoding the selectable marker, wherein recombination of the two first recombining sites produces a nucleic acid sequence that cannot be transcribed to produce a functional protein.

There is no motivation or suggestion, in either Rajewsky et al, Muyrers et al. or Stewart et al., to use methods for generating vectors for a conditional knock out that included the use of two selectable markers, each flanked by a pair of recombining sites.

As discussed above, these elements must be present to support a finding of obviousness there must be: (1) a finding that the prior art included each element claimed, although not necessarily in a single prior art reference, with the only difference between the claimed invention and the prior art being the lack of actual combination of the elements in a single prior art reference; (2) a finding that one of ordinary skill in the art could have combined the elements as claimed by known methods, and that in combination, each element merely performs the same function as it does separately; and (3) a finding that one of ordinary skill in the art would have recognized that the results of the combination were predictable (MPEP § 2143). This burden has not been met in the present case: (1) the prior art does not include each element claimed, as none of Rajewsky et al., Muyrers et al. or Stewart et al. describe methods that include either the introduction of a second selectable marker or a second pair of recombining sites; (2) the prior art could not have combined the elements as claimed by known methods, since Rajewsky et al. only describe homologous recombination in ES cells with a targeting vector that only includes three recombining sites (two of these flanking a single selectable marker), and Stewart et al. and Muyrers et al. teach recombination methods that are functional only in bacterial cells (not ES cells) and Muyrers et al. teach away from the use of recombining sites; and (3) there is no evidence to support that the claimed methods would be predictable (especially in view of Muyrers et al.) absent the present disclosure (which simply cannot be used as part of the obviousness rejection).

Reconsideration and withdrawal of the rejection are respectfully requested.

With regard to both of the rejections based on Rajewsky et al., (in combination with Muyrers et al. and Stewart et al. and/or Lee et al.) there is evidence presented in the specification documenting the unexpected superior properties of the claimed methods. Specifically, none of the cited prior art discloses a vector comprising the BAC. Rajewsky et al. describe the use of targeting vectors, but do not describe the use of BACs. In Muyrers et al. the target molecules is described as being an episome that is either a "plasmid or a bacterial artificial chromosome" (see the legend of Fig. 1). Similarly, Stewart et al. disclose that the target DNA can be a BAC, plasmid or E. coli chromosome (see section 5.2.4). Lee et al. describe methods for targeting or subcloning a BAC. However, the specification discloses that the claimed methods are very rapid and efficient. Specifically, it is disclosed on page 112, line 17-25 that "By using high copy plasmid DNA for vector construction, the problem caused by Lox sites present in the BAC vector backbone is eliminated..." and that using the claimed methods with long homology arms "targeting frequencies as high as $1X10^{-2}$ can be obtained with as little as 100 ng of targeting DNA (i.e., targeting a floxed *Neo* cassette to a BAC)." Using the claimed methods, more than ten targeting vectors were produced (see the specification at page 113, lines 21-26); all of these vectors were highly efficient in targeting frequencies in mouse ES cells. Using embodiments of the claimed methods it takes less than two weeks to construct a cko-targeting vector using this method, and multiple cko vectors can be generated simultaneously (see page 114).

This showing of an unexpectedly superior result overcomes any *prima facie* case of obviousness based on Rajewsky et al., Lee et al., Muyrers et al, and/or Stewart et al. in any combination.

Conclusion and Formal Request for Interview

Applicants believe the present application is ready for allowance, which action is requested. If any issues remain prior to the issuance of a Notice of Allowance, or if the Examiner has any questions regarding Declaration of Dr. Liu under 37 C.F.R. § 1.132 or the Declaration under 37 C.F.R. § 1.131, the Examiner is formally requested to contact the undersigned using the telephone number provided below prior to issuance of the next Office Action in order to arrange a telephonic interview. It is believed that a brief discussion of the merits of the present application may expedite prosecution. This request is being submitted under MPEP § 713.01, which indicates that an interview may be arranged in advance by a written request.

Respectfully submitted,

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